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(54) Title: TREATMENT OF AUTOIMMUNE DISEASE USING ORAL TOLERIZATION AND/OR Th2-ENHANCING CYTOKINES

(57) Abstract

The invention is directed to methods for treating autoimmune diseases such as multiple sclerosis by orally administering a bystander antigen such as myelin basic protein or proteolipid protein in conjunction with a non-interferon polypeptide having Th2-enhancing cytokine activity in such a manner as to induce oral tolerance to the bystander antigen resulting in suppression of the autoimmune response.

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TREATMENT OF AUTOIMMUNE DISEASE USING ORAL TOLERIZATION AND/OR Th2-ENHANCING CYTOKINES

FIELD OF THE INVENTION

This invention pertains to an improvement in the ability to reduce autoimmune reactions by use of oral tolerization. More specifically, the invention is directed to the oral (or by-inhalation) administration of autoantigens or, more generally, bystander antigens in combination with oral administration of polypeptides having Th2-enhancing cytokine activity for reducing autoimmune reactions or responses.

Another aspect of the invention pertains to oral use of Th2-enhancing cytokines (i.e. cytokines that bias the immune system towards a Th-2 response either by increasing Th-2 responses or by inhibiting Th-1 responses) in reducing autoimmune reactions.

BACKGROUND OF THE INVENTION

Autoimmune diseases are characterized by an abnormal immune response directed against normal autologous (self) tissues.

Based on the type of immune response (or immune reaction) involved, autoimmune diseases in mammals can generally be classified in one of two different categories: cell-mediated (i.e., T-cell-mediated) or antibody-mediated disorders. Non-limiting examples of cell-mediated autoimmune diseases include multiple sclerosis (MS), rheumatoid arthritis (RA), autoimmune thyroiditis (AT), the autoimmune stage of diabetes mellitus (juvenile-onset or Type 1 diabetes) and autoimmune uveoretinitis (AUR). Antibody-mediated autoimmune

diseases include without limitation myasthenia gravis (MG) and systemic lupus erythematosus (SLE).

Both categories of autoimmune diseases are currently being treated with drugs that suppress immune responses systemically in a non-specific manner, i.e., drugs incapable of selectively suppressing the abnormal immune response. Non-limiting examples of such drugs include methotrexate, cyclophosphamide, Imuran (azathioprine) and cyclosporin A. Steroid compounds such as prednisone and methylprednisolone (also non-specific immunosuppressants) are also employed in many instances. All of these currently employed drugs have limited efficacy against both cell- and antibody-mediated autoimmune diseases. Furthermore, such drugs have significant toxic and other side effects and, more important, eventually induce "global" immunosuppression in the subject being treated. In other words, prolonged treatment with the drugs downregulates the normal protective immune response against pathogens thereby increasing the risk of infection. In addition, patients subjected to prolonged global immunosuppression have an increased risk of developing severe medical complications from the treatment, such as malignancies, kidney failure and diabetes.

In a continuing effort to overcome the drawbacks of conventional treatments for autoimmune disease, the present inventors and their coworkers have devised methods and pharmaceutical formulations useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments are based on the concept of inducing tolerance, orally or by inhalation, using as the tolerizers autoantigens or bystander antigens or disease-suppressive fragments or analogs of autoantigens or bystander antigens. This body of work has been described in co-pending PCT Patent Applications Nos. PCT/US93/01705 filed February 25, 1993, PCT/US91/01466 filed March 4, 1991, PCT/US90/07455 filed December 17, 1990, PCT/US90/03989 filed July 16, 1990, PCT/US91/07475 filed October 10, 1991, PCT/US93/07786 filed August 17, 1993, PCT/US93/09113 filed

September 24, 1993, PCT/US91/08143 filed October 31, 1991, PCT/US91/02218 filed March 29, 1991, PCT/US93/03708 filed April 20, 1993, PCT/US93/03369 filed April 9, 1993, and PCT/US91/07542 filed October 15, 1991.

Autoantigens and bystander antigens are defined below.

Intravenous administration of autoantigens and preferably fragments thereof consisting essentially of immunodominant epitopic regions of their molecules has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy, or T-cell nonresponsiveness, causes deactivation of immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, such as myelin basic protein (MBP), once anergized, no longer proliferate in response to that antigen. The inability of the anergized T-cells to proliferate results in a reduction of the immune attack reactions that cause the tissue damage responsible for the autoimmune disease symptoms, such as the neural tissue damage observed in MS. There is also evidence that oral administration of autoantigens or immunodominant fragments thereof in a single dose and in substantially larger amounts than those that trigger active suppression may also induce tolerance through anergy (or clonal deletion).

Clonal anergy, however, can be induced only when the administered antigen is the specific antigen recognized by the immune attack T-cells sought to be anergized (pure bystander antigens do not induce tolerance through anergy). Thus, regimes that rely on clonal anergy to achieve suppression have certain limitations: the autoantigen may not be known, or there may be several types of immune attack T-cells specific to different antigens, or the antigens to which the immune attack T-cells are specific may change over time.

The present inventors and their co-workers have developed a method of treatment that uses autoantigens and proceeds by active suppression, a different mechanism than clonal anergy. This method, discussed extensively in the

related PCT Application PCT/US93/01705, involves the oral administration of antigens specific to the tissue under autoimmune attack. These are called "bystander antigens" and are defined below. This treatment causes regulatory (suppressor) T-cells to be induced in the gut-associated lymphoid tissue (GALT), or, in the case of by-inhalation administration, mucosa associated lymphoid tissue (MALT). These regulatory cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted with the autoimmune disease and which suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one antigenic determinant of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of certain immunomodulatory factors and cytokines, such as transforming growth factor beta (TGF-β) interleukin-4 (IL-4) or interleukin-10 (IL-10). Of these, TGF-β is an antigen-nonspecific immunosuppressive factor in that it suppresses all immune attack phenomena regardless of the antigen that triggers these phenomena. (However, because oral tolerization with a bystander antigen causes the release of TGF-β only in the vicinity of autoimmune attack, no systemic immunosuppression ensues.) IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances Th2 response, i.e. acts on T-cell precursors and causes them to differentiate preferentially into Th2 cells. IL-4 also indirectly inhibits Th1 exacerbation. IL-10 is a direct inhibitor of Th1 responses.

After orally tolerizing mammals afflicted with an autoimmune disease conditions with bystander antigens, the present inventors and their co-workers observed increased levels of TGF-β, IL-4 and IL-10 at the locus of autoimmune attack. Chen, Y. et al., Science, 265:1237-1240, 1994.

Recently, the present inventors and their coworkers have found that oral or parenteral administration of Type I interferon, or polypeptides having Type I interferon activity, either alone or in conjunction with oral or by-inhalation administration of autoantigens or bystander antigens, is

beneficial in reducing the symptoms of autoimmune disease. In fact, even suboptimal doses of Type I interferon potentiate the tolerizing effect of the autoantigens and bystander antigen. This work has been described in more detail in one of the priority documents of this application. Type I interferon, especially β-IFN, is known to have certain immunomodulatory properties e.g. inhibition of the activity of γ-interferon (IFN-γ). IFN-γ has been shown to exacerbate MS, and may be involved in the pathogenesis of MS lesions. Thus, IFN-β appears to have an effect due in part to its ability to inhibit IFN-γ expression by T-cells. The related ability of IFN-β polypeptide to reduce expression of class II major histocompatibility complex (MHC) molecules on T-cell surfaces, as well as the ability to increase activity of suppressor T-cells, are also thought to be responsible for the tolerance-promoting immunomodulatory properties of IFN-β used parenterally.

The mechanism by which orally administered IFN-β promotes tolerance, either alone or as a synergist when used in conjunction with an antigen, is not well understood (for example it is not known whether interferon-β is a Th2-enhancing cytokine; in any event it is not the subject of the present invention). The polypeptide is neither an autoantigen nor a bystander antigen. Furthermore, the effect of a substance on the immune system of a mammal (as well as the mechanisms by which these effects are brought about) is often different depending on the amount and/or route of administration of that substance. For example, subcutaneous administration of an alloantigen induces an immune response to that antigen. Oral administration of the same alloantigen may induce tolerance by eliciting T-suppressor cells that are specific to the orally administered antigen or (with higher doses and infrequent administration) may induce anergy. Intravenous administration of the same alloantigenic substance may induce tolerance by way of anergy.

To date there has been no teaching of oral (or parenteral) use of immunomodulatory (e.g. Th2-enhancing) cytokines such as IL-4 in the treatment of autoimmune disease.

Further, there has been no teaching of oral (or parenteral) use of Th2-enhancing cytokines in conjunction with oral tolerization employing autoantigens or bystanders antigens.

Accordingly, one object of the present invention is to provide an improved and/or more convenient method for treating mammals suffering from autoimmune diseases.

An additional object of the present invention is an improved method for treating mammals suffering from autoimmune diseases exclusively via the oral route.

A third object of the invention is a method for treating mammals suffering from autoimmune diseases through the oral administration of immunomodulatory cytokines.

SUMMARY OF THE INVENTION

It has now been surprisingly found that:

- oral or by-inhalation administration of IL-4 and peptide or polypeptide fragments thereof having Th2-enhancing cytokine activity is of benefit in the abatement (suppression) of autoimmune reactions associated with autoimmune disease;
- a combination of (i) oral or by-inhalation administration of autoantigens or bystander antigens (or fragments of them) and (ii) administration of polypeptides having Th2-enhancing cytokine activity is substantially more effective than the use of autoantigens or bystander antigens alone (or of the Th2-enhancing cytokines alone) in suppressing autoimmune reaction associated with autoimmune diseases.

In addition, parenteral administration of IL-4 both has a suppressing effect on autoimmune reaction associated with autoimmune disease and enhances the suppressive effect of bystander antigens.

Use of other noncytokine synergists can be further conjoined to the foregoing combination.

DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of conflict, the

description including the definitions and interpretations of the present disclosure will prevail.

Definitions

The following terms, when used in this disclosure, shall have the meanings ascribed to them below:

"Th2-enhancing cytokines" are naturally occurring antigen-nonspecific immunoregulatory substances that: (i) are normally secreted or induced by regulatory immune system cells and (ii) enhance the frequency of Th2 cells (and/or inhibit Th1 cells).

It has now surprisingly been found that oral administration of Th2-enhancing cytokines, either alone or in conjunction with bystander antigens (see below), is beneficial in reducing autoimmune reactions or responses. Non-limiting examples of such cytokines include IL-4, and fragments thereof that retain Th2-enhancing activity. It should be noted however, that it is not at this time known whether the tolerizing effect observed by oral administration of IL-4 and the tolerization enhancing effect observed by administering IL-4 in conjunction with a bystander antigen are due to the Th2-enhancing properties of IL-4.

"Bystander antigen" or "bystander" is a protein, protein fragment, peptide, glycoprotein, or any other immunogenic substance (i.e. a substance capable of eliciting an immune response) that (i) is, or is derived from, a component specific to the organ or tissue under autoimmune attack; and (ii) upon oral or enteral administration elicits regulatory (suppressor) T-cells (which can be of the CD4+ or CD8+ type) that are targeted to the organ or tissue under attack where they cause at least one antigen-nonspecific immunosuppressive factor or immunoregulatory cytokine (such as TGF-β, IL-4 or IL-10) to be released and thereby suppress immune attack cells that contribute to autoimmune destruction. The term includes but is not limited to autoantigens and fragments thereof involved in autoimmune attack. In addition, the term includes antigens normally not exposed to the immune system which become exposed in the locus of autoimmune attack as a result of autoimmune tissue destruction. An example is

heatshock proteins, which although not specific to a particular tissue are normally shielded from contact with the immune system.

"Bystander suppression" is suppression at the locus of autoimmune attack of cells that contribute to autoimmune destruction; this suppression is mediated by the release of one or more immunosuppressive factors (including Th2-enhancing cytokines and Th1-inhibiting cytokines) from suppressor T-cells elicited by the ingestion (or inhalation) of a bystander antigen and recruited to the site where cells contributing to autoimmune destruction are found. The result is antigen-nonspecific but locally restricted downregulation of the autoimmune responses responsible for tissue destruction.

"Mammal" is defined herein as any organism having an immune system and being susceptible to an autoimmune disease.

"Autoimmune disease" is defined herein as a spontaneous or induced malfunction of the immune system of mammals, including humans, in which the immune system fails to distinguish between foreign immunogenic substances within the mammal and/or autologous substances and, as a result, treats autologous tissues and substances as if they were foreign and mounts an immune response against them. The term includes human autoimmune diseases and animal models therefor.

"Autoantigen" is any substance or a portion thereof normally found within a mammal that, in an autoimmune disease, becomes the primary (or a primary) target of attack by the immunoregulatory system. The term also includes antigenic substances that induce conditions having the characteristics of an autoimmune disease when administered to mammals. Additionally, the term includes peptic subclasses consisting essentially of immunodominant epitopes or immunodominant epitope regions of autoantigens. Immunodominant epitopes or regions in induced autoimmune conditions are fragments of an autoantigen that can be used instead of the entire autoantigen to induce the disease. In humans afflicted with an autoimmune disease, immunodominant epitopes or regions are fragments of antigens specific to the tissue or organ under autoimmune attack and recognized by a substantial percentage (e.g. a

majority though not necessarily an absolute majority) of autoimmune attack T-cells.

"Treatment" is intended to include both the prophylactic treatment to prevent or delay the onset of an autoimmune disease (or to prevent the manifestation of clinical or subclinical, e.g., histological, symptoms thereof), as well as the therapeutic suppression or alleviation of symptoms after the manifestation of such autoimmune disease, by abating autoimmune attack and preventing or slowing down autoimmune tissue destruction. "Abatement", "suppression" or "reduction" of autoimmune attack or reaction encompasses partial reduction or amelioration of one or more symptoms of the attack or reaction. A "substantially" increased suppressive effect (or abetment or reduction) of autoimmune reaction means a significant decrease in one or more markers or histological or clinical indicators of autoimmune reaction or disease. Nonlimiting examples are a reduction by at least 1 unit in limb paralysis score or in arthritis score or a significant reduction in the frequency of autoreactive T-cells; a reduction of at least about 0.5 units in insulitis scoring (measured e.g. as described in Zhang et al., PNAS, 1991, 88:10252-10256).

As used in the present specification, administration of a Th2-enhancing cytokine "in conjunction with" (or "in association with") bystander antigens means before, substantially simultaneously with, or after oral (or by-inhalation) administration of bystander antigens. "Substantially simultaneously" means within the same 24-hour period, or preferably within one hour before or after bystander administration.

"Oral" administration includes oral, enteral or intragastric administration. In addition, by-inhalation administration in aerosol form accomplishes the same tolerizing effect and is equivalent to oral tolerization.

"Parenteral" administration includes subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal or intrathecal administration.

Animal Models

Throughout the present specification, reference is made to various model systems that have been developed for studying autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE) has been studied in mice and other rodent species as a model for Multiple Sclerosis (MS). Those of ordinary skill in the art recognize that many of the potential immune therapies for MS are first tested in this animal model system. The disease is induced by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) and an adjuvant (such as Freund's Complete Adjuvant, "FCA"). The antigen that is used to induce the disease is the autoantigen in the model. This treatment, with either antigen, induces either a monophasic or an exacerbating/remitting form of demyelinating disease (depending on the type and species of rodent and well-known details of induction). The induced disease has many of the characteristics of the autoimmune disease MS and serves as an animal model therefor. Furthermore, the successful treatment of EAE by oral tolerization, and the parallel success in decreasing the frequency of disease-inducing cells in humans, and, in many cases, ameliorating the symptoms of MS, using oral administration of myelin, validates the use of EAE as a model system for predicting the success of different oral tolerization regimens. Immunization with Mycobacterium tuberculosis or with Freund's Complete Adjuvant in oil into the dorsal root tail of susceptible mammals induces a disease used as a model for human rheumatoid arthritis. In like manner, immunization with Type II collagen with an adjuvant will also induce a disease (collagen-induced arthritis or "CIA") that serves as a model for human rheumatoid arthritis. These animal models also serve as good predictors of successful oral tolerization using bystander antigens.

Immunization of Lewis rats with S-antigen or IRBP-antigen (InterPhotoReceptor Binding Protein) and an adjuvant induces autoimmune uveoretinitis. Finally, a model for Type I diabetes develops spontaneously in the NOD Mouse.

One or more of the above disclosed model systems may be employed to demonstrate the efficacy and improved treatment

provided by the present invention. In fact, the animal models are particularly suitable for testing therapies involving bystander suppression, precisely because this suppression mechanism is antigen-nonspecific. In the case of oral tolerization, therefore, the suppression of symptoms obtained in the model is independent of many of the actual or potential differences between a human autoimmune disorder and an animal model therefor. The same animal models are suitable for testing therapies based on use of Th2-enhancing cytokines because the cytokines generally have the same or similar activities in animal models as in humans.

The above animal models can be thus used to establish the utility of the present invention in mammals (including humans). For example, a multiple sclerosis autoantigen, bovine myelin, orally administered to humans in a double-blind study conferred a considerable benefit to a significant patient subset (Weiner, H. et al. Science 259:1321-1324, 1993). In addition, rheumatoid arthritis symptoms, such as joint tenderness, AM stiffness, grip strength, etc., were successfully suppressed in humans receiving oral collagen (0.1-0.5 mg single dose daily). (Trentham, D. et al., Science 261:1727, 1993.) Finally, preliminary human trials with oral S-antigen showed very encouraging results for uveoretinitis. Large scale human studies are presently conducted for multiple sclerosis, uveoretinitis, rheumatoid arthritis and diabetes. All of these human trials now validate the animal data on oral tolerization using the appropriate disease model. Thus, the predictive value of animal models for oral tolerization treatment of autoimmune diseases is substantially supported by these human clinical studies.

What follows is a description of the individual treatments that have now been combined in the treatment method of the present invention. By describing the effect of each of the possible treatments individually, followed by a discussion of the combination treatment, the present specification allows one of ordinary skill to understand the efficacy of these treatments, when combined, to reduce or eliminate tissue damage in autoimmune disease.

Description of Bystander Suppression--Oral Administration

In contrast to clonal anergy, suppression mediated by oral (or by-inhalation) administration of bystander antigens is brought about by elicitation of targetable immunoregulatory T-cells that release one or more immunosuppressive factors, such as transforming growth factor-beta (TGF-β); and/or Th2-enhancing cytokines, such as interleukin 4 (IL-4); and/or interleukin 10 (IL-10) at the locus of the autoimmune attack. These regulatory T-cells do not release high levels of IL-2 or γ-IFN. Because regulatory T-cells are elicited, the mechanism at work is referred to as active suppression. The immunoregulatory cytokines released by the elicited regulatory cells are antigen-nonspecific, even though these regulatory T-cells release (or induce the release of) immunoregulatory cytokines only when triggered by an antigenic determinant identical to one on the orally administered (or inhaled) antigen. Recruitment of the immunoregulatory T-cells to a locus within a mammal where cells contributing to the autoimmune destruction of an organ or tissue are concentrated allows for the release of immunoregulatory substances in the vicinity of the autoimmune attack and suppresses all types of immune system cells responsible for such attack.

Because the T-suppressor cells have been elicited in response to oral (or by-inhalation) tolerization with a tissue- or organ-specific antigen, the target for the suppressor T-cells is the organ or tissue under immune attack in the particular autoimmune disease where the destructive cells will be concentrated. Thus, the bystander antigen may be an autoantigen or an immunodominant epitope of an autoantigen. Alternatively, the bystander may be another tissue-specific antigen that is not an autoantigen; hence, the autoantigen (or autoantigens) involved need not be identified.

In more detail, an example of the active suppression mechanism of bystander suppression for a tissue-specific (bystander) antigen is as follows: After a tissue-specific (bystander) antigen is administered orally (or enterally, i.e., directly into the stomach) it passes into the small intestine, where it comes into contact with the so-called Peyer's patches

and villi, which are collections of a large number of immunocytes located under the intestinal wall. These cells, in turn, are in communication with the immune system, including the spleen and lymph nodes. The result is that suppressor (CD8+ or CD4+) T-cells are induced, released into the blood or lymphatic circulation, and then recruited to the area of autoimmune attack, where they cause the release of TGF-β and/or other immunoregulatory substances that downregulate the activated helper T-cells as well as the B-cells directed against the mammal's own tissues. Chen, Y. et al., Science, 1994 supra. Suppression induced in this manner is antigen-nonspecific. However, the resulting tolerance is specific for the particular autoimmune disease, i.e. for a particular tissue under autoimmune attack, by virtue of the fact that the bystander antigen is specific for the tissue under attack and suppressor cells elicited by ingestion of the bystander antigen suppress the immune attack cells that are found at or near the tissue being damaged.

Bystander antigens and autoantigens (as well as fragments and analogs of any of them) can be purified from natural sources (the tissue or organ where they normally occur) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual bystander antigens are known: See, e.g., Hunt, C. et al PNAS (USA), 82:6455-6459, 1985 (heat shock protein hsp70); Burkhardt, H., et al., Eur. J. Immunol. 21:49-54, 1991 (antigenic collagen II epitope); Tuohy, V.K., et al., J. Immunol. 142:1523-1527, 1989 (encephalitogenic determinant of mouse PLP in mice); Shinohara, T. et al., In Progress in Retinal Research, Osborne, N. & Chader, J. Eds, Pergamon Press 1989, pp. 51-55 (S-antigen); Donoso, L.A., et al., J. Immunol. 143:79-83, 1989 (IRBP); Borst, D.E., et al., J. Biol. Chem. 264:115-1123, 1989 (IRBP); Yamaki, K. et al., FEBS 234:39-43, 1988 (S-antigen); Donoso, L.A. et al., Eye Res. 7:1087, 1988 (IRBP); Wyborski, R.J., et al., Mol. Brain Res. 8:193-198, 1990 (GAD).

The amino acid sequences for bovine and mouse PLP; bovine, human, chimpanzee, rat, mouse, pig, rabbit, guinea pig MBP; human and bovine collagen alpha-1(II) and bovine collagen alpha-1(I); and human insulin are well-known and published and these antigens can be synthesized by recombinant techniques, as is well-known in the art. Fragments of these antigens can be chemically synthesized or also synthesized by recombinant techniques.

Some tissue-specific antigens are commercially available: e.g. insulin, glucagon, myelin basic protein, myelin, collagen I, collagen II, proteolipid protein, etc.

Bystander antigens can be identified with routine experimentation. Any antigen from the afflicted tissue is a potential bystander. The potential bystander can be fed to mammals, and spleen cells or circulating T-cells from, e.g. the blood or cerebrospinal fluid in the case of EAE or MS, from these mammals can be removed and stimulated in vitro with the same antigen. T-cells elicited by stimulation can be purified and supernatants can be tested for their content of TGF-β, IL-4, IL-10, or other immunoregulatory substances. In particular, TGF-β can be measured quantitatively and/or qualitatively by ELISA using preferably a suitable commercially available polyclonal or most preferably monoclonal antibody raised against TGF-β (e.g. R&D Systems, Minneapolis, MN; Celtrix Pharmaceuticals, Santa Clara, CA). Miller, A. et al., J. Immunol., 148:1106, 1992. Alternatively, another known assay for TGF-β detection can be employed, such as that described in Example 2 below using a commercially available mink lung epithelial cell line. If the bystander antigen elicits T-suppressor cells that do not release TGF-β, the T-cells can be similarly tested for secretion of IL-4 or IL-10 (antibodies for IL-4 and IL-10 are commercially available, e.g. from Pharmingen, San Diego, CA). Tissue-specific antigens that are not effective bystanders are those so segregated from the inflammatory locus (of autoimmune attack) so that the immunoregulatory cytokines released will be too far removed from the locus of inflammation to exert a suppressive effect.

The efficacy of orally induced bystander suppression can be assessed, e.g., by: diminution in certain inflammation markers, such as the number of activated T-cell clones directed against the organ or tissue that is the target of autoimmune attack; decrease in IL-2 or IFN-γ levels at the same locus; histological evaluation of the afflicted organ or tissue (e.g., by biopsy or magnetic resonance imaging); or reduction in the number and/or severity of clinical symptoms associated with an autoimmune disease.

Use of Bystander Antigens - Dosages

The tolerance induced by the bystander antigens of this invention is dose-dependent over a broad range of oral (or enteral) or inhalable dosages. However, there are minimum and maximum effective dosages. In other words, active suppression of the clinical and histological symptoms of an autoimmune disease occurs within a specific dosage range, which, however, varies from disease to disease, mammal to mammal, and bystander antigen to bystander antigen. For example, when the disease is PLP-induced EAE in mice, the suppressive dosage range when MBP is used as the bystander is from about 0.1 to about 1 mg/mouse/feeding (with feedings occurring about every other day e.g., 5-7 feedings over a 10-14-day period). A most preferred dosage is 0.25 mg/mouse/feeding. For suppression of the same disease in rats, the MBP suppressive dosage range is from about 0.5 to about 2 mg/rat/feeding and the most preferred dosage is 1 mg/rat/feeding. The effective dosage range for humans with MS, when MBP is used as the oral tolerizer, is between about 1 and about 100, preferably between about 1 and about 50 mg MBP per day (administered every day or on alternate days for a period of time ranging from several months to several years) with the optimum being about 30 mg/day.

For rheumatoid arthritis, the effective dosage range for humans receiving either Type I or II or Type III collagen is about 0.1 to about 1 mg/day, and preferably 0.1-0.5 mg/day. For adjuvant-induced arthritis in rats, the effective collagen dosage range is about 3 to about 30 micrograms/feeding with the same feeding schedule as for EAE.

Monitoring of the patient may be desirable in order to optimize the dosage and frequency of administration. The exact amount and frequency of administration to a patient may vary depending on the stage, frequency of manifestation and severity of the patient's disease and the physical condition of the patient, as is well-appreciated in the art. Such optimization is preferably effected on a case-by-case basis. Optimization of the dosage necessary for immune suppression involves no more than routine experimentation, given the guidelines disclosed herein.

Assessment of the disease severity can be accomplished according to well-known methods depending on the type of disease. Such methods include without limitation:

MS: severity and number of attacks over a period of time; progressive accumulation of disability (which can be measured, e.g. on the Expanded Disability Status Scale); number and extent of lesions in the brain (as revealed, e.g., by magnetic resonance imaging); and frequency of autoreactive T-cells.

EAE: limb paralysis which can be scored as follows: 0-no disease; 1-decreased activity, limp tail; 2-mild paralysis, unsteady gait; 3-moderate paraparesis, limbs splayed apart; 4-tetraplegia; and 5-death.

RA: joint swelling, joint tenderness, morning stiffness, grip strength, joint imaging techniques.

AUR: visual acuity; number of T-cells in the eye and "cloudiness" in the eye.

Type I Diabetes: pancreatic beta cell function (assessed, e.g., by OGTT glucose tolerance test).

NOD Model: insulitis and delay of diabetes onset.

CIA: Arthritis score based on number of affected joints in each of four paws and grading each on an arbitrary scale of 1-4 as follows: 0=normal; 1=redness only; 2=redness plus

swelling; 3=severe swelling; and 4=joint deformity. The total arthritis score is the sum of the scores for all paws. Maximum arthritis score is the highest score for an animal over the course of the disease. According to this grading method the highest arthritis score possible is 16 (4 paws X 4 score-per-paw).

Stabilization of symptoms, under conditions wherein control patients or animals experience a worsening of symptoms, is one indicator of efficacy of a suppressive treatment. Another measure of improvement is the ability to reduce or discontinue other medications, e.g., steroids or other anti-inflammatory medications, and biologic response modifiers such as methotrexate, subcutaneous interferon and the like. The optimum dosage of a bystander antigen will be the one generating the maximum beneficial effect assessed as described above. An effective dosage will be one that causes at least a statistically or clinically significant attenuation of at least one marker, symptom or histological evidence characteristic of the disease being treated as described above. (Clinically significant-attenuation is one observed by a clinician of ordinary skill in the field of a particular autoimmune disease.)

When combined with IL-4 treatment, the dosage of bystander antigen should be preferably equal to that which would have been used if oral or enteral administration of the bystander antigen was used alone, except that the combination is more effective in abating autoimmune reaction. However, the level of IL-4 can be the same as that when IL-4 is used alone, or suboptimal (i.e. an amount which would not be effective if IL-4 were used alone but is nevertheless sufficient to potentiate the tolerizing effect of the bystander antigen.

Ascertaining the effective dosage range as well as the optimum amount of bystander antigen is well within the skill in the art. For example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose (e.g., 1 microgram), progressively increasing it (e.g.

logarithmically) and measuring the number of TGF-beta (and/or IL-4 or IL-10) secreting cells and/or assessing the number and activation of immune attack T-cells in the blood (e.g. by limiting dilution analysis and ability to proliferate) and/or assessing the disease severity, as described above. The optimum dosage will be the one generating the maximum amount of suppressive cytokines in the blood and/or causing the greatest decrease in disease symptoms. An effective dosage range will be one that causes at least a statistically or clinically significant attenuation of at least one symptom characteristic of the disease being treated.

The maximum effective dosage of a bystander can be ascertained by testing progressively higher dosages in animals and then extrapolating to humans. For example, based on the dosages given above, for rodents, the maximum effective dose of MBP for humans has been estimated between 50 and 100 mg/feeding. Similarly, the maximum effective amount of Collagen Type II for humans has been estimated at about 1 mg/day.

The present invention can also be advantageously used to prevent the onset of an autoimmune disease in susceptible individuals at risk for an autoimmune disease. For example, methods for the identification of patients who are at risk for developing Type 1 diabetes are extant and reliable and have been recently endorsed by the American Diabetes Association (ADA). Various assay systems have been developed which (especially in combination) have a high predictive value assessing susceptibility to Type 1 diabetes (Diabetes Care 13: 762-775, 1990). Details of one preferred screening test are available to those of ordinary skill in the art (Bonifacio, E. et al., The Lancet 335: 147-149, 1990).

From a practical point of view, preventing the onset of most autoimmune diseases is of most importance in the case of diabetes. Other autoimmune diseases MS, RA, AT and AUR are declared at an earlier stage of tissue destruction, before substantial tissue damage has taken place; therefore preventive treatment of these diseases is not as important as in the case of diabetes. In diabetes, it would be best to intervene with

an effective treatment prior to the substantial destruction of substantially all of the pancreatic islet cells. After the islet cells are destroyed, the treatment would not be effective.

A non-limiting list of autoimmune diseases and tissue- or organ-specific confirmed or potential bystander antigens effective in the treatment of these diseases when administered in an oral or inhalable form are set forth in Table 1 below. Administration of combinations of antigens listed for each individual disease (alone or in conjunction with IL-4) is also expected to be effective in treating the disease.

Bystander antigens and Th2-enhancing cytokines can be also administered by inhalation. The bystander amounts that need to be inhaled are generally smaller than those for oral administration. It is anticipated that the amounts of Th2-enhancing cytokines administered by inhalation will be likewise smaller. Effective amounts for inhalation therapy can be assessed using the same methodologies provided above.

Table 1

Autoimmune Disease	Affected Tissue	Bystander Antigen	Source	Type
Type 1 Diabetes	pancreatic beta cells	glucagon insulin GAD		
Multiple Sclerosis	myelinated neurons	MBP, MBP fragments PLP, PLP fragments myelin-assoc GP myelin oligodendrocyte glycoprotein; heatshock protein	J.Chromatog. Biomed.Appl. 526:535 (90)	purification
Rheumatoid Arthritis	connective tissue	collagen I, II or III RO/SS-A RO/SS-B-LA heatshock protein	J.Immunol.Meth121:219 (89) 151:177 (92)	purification purification
Autoimmune uveitis	eye	S-antigen IRBP recoverin	Exp.Eye Res. 56:463 (93)	cDNA
Myasthenia Gravis	muscle	acetylcholine receptor heatshock protein	Eur.J.Pharm. 172:231 (89)	purification
Male Infertility	sperm	NASP(post-acrosomal sperm protein)	Biol.Reprod. 43:559 (90)	cDNA
Myositis	muscle	Jo-1 antigen heatshock protein	Biol.Chem.H-S. 368:531 (87)	purification

Autoimmune Disease	Affected Tissue	Bystander Antigen	Source	Type
Pemphigus	skin	desmoglein Factor XIII	Eur.J.Cell Biol. 55:200 (91)	cDNA

For any autoimmune disease, extracts of the relevant tissue, as well as specific bystander antigens or fragments thereof, can be used as oral tolerizers. In other words, the bystander antigen need not be purified. For example, myelin (which could be derived from different species) has been used for MS, pancreatic cell extracts have been used for Type 1 diabetes, splenic cell extracts have been used to prevent allograft rejection (which is not, strictly speaking, an autoimmune phenomenon), and muscle extracts have been used to treat myositis. However, administration of one or more individual antigens or fragments is preferred.

Thus, according to the present invention, when treating Type 1 diabetes, an effective amount (determined as described above) of glucagon can be administered orally. Glucagon is specifically present in the pancreas. Glucagon, however, is clearly not an autoantigen because it is not expressed in pancreatic beta cells which are destroyed in the course of Type 1 diabetes (glucagon is found exclusively in alpha cells, a different cell type). Thus, glucagon is a "pure" bystander: it does not appear to have any autoantigen activity. (Presumably, the bystander activity of glucagon results from its high local concentration in the pancreatic intercellular milieu due to its secretion from alpha cells.)

Insulin has bystander activity for Type 1 diabetes. It is not at present known whether insulin is also an autoantigen, although anti-insulin autoantibodies are found in Type 1 patients. However, whatever the mechanism of action, oral, enteral or inhalable insulin preparations are effective in suppressing Type 1 diabetes and animal models therefor by preventing autoimmune destruction of pancreatic beta cells.

For multiple sclerosis and animal models therefor, both disease inducing and noninducing fragments of MBP have bystander activity not only for MBP-induced disease but also for PLP-induced disease. In rats, feeding of bystander generates mostly $CD8^+$ suppressor cells which are class I restricted, whereas in mice both $CD8^+$ suppressors and $CD4^+$ regulatory cells are generated (the latter being probably Class II restricted). Chen, Y. et al. <u>Science</u>, 1994, <u>supra</u>.

For rheumatoid arthritis and animal models therefor, Type-I, Type-II and Type-III collagen have activity as oral tolerizers. Other collagens are likely to be similarly active.

For uveoretinitis and its animal model, S-antigen and IRBP and fragments thereof have bystander activity.

Fragments of bystander antigens can also be employed. Useful fragments can be identified using the overlapping peptide method and T-cells from fed animals can be tested for secretion of TGF-β, and/or IL-4 and/or IL-10, and can further be identified by subtype ($CD8^+$ and/or $CD4^+$).

Orally administered autoantigens and bystander antigens elicit regulatory T-cells and thereby induce the production and/or release of TGF-β and/or IL-4 and IL-10. One such T-cell has been identified in mice orally tolerized against EAE as a CD4+ suppressor T-cell, and a CD8+ suppressor T-cell has been identified in rats. Even immunodominant epitopes of autoantigens, e.g. MBP are capable of inducing such regulatory T-cells. Additional such epitopes can be identified by feeding a bystander antigen to a mammal and isolating from the mammal T-cells that recognize a fragment of the antigen (and thus identifying suppressive fragments), or by identifying T-cells from a bystander fed mammal that can adoptively transfer protection to naive (not-fed) animals.

The bystander antigens can be administered alone or in conjunction with autoantigens. Autoantigen administration is carried out as disclosed in PCT Applications PCT/US93/01705 filed February 25, 1993, PCT/US91/01466 filed March 4, 1991, PCT/US90/07455 filed December 17, 1990, PCT/US90/03989 filed July 16, 1990, PCT/US91/07475 filed October 10, 1991, PCT/US93/07786 filed August 17, 1993, PCT/US93/09113 filed September 24, 1993, PCT/US91/08143 filed October 31, 1991, PCT/US91/02218 filed March 29, 1991, PCT/US93/03708 filed April 20, 1993, PCT/US93/03369 filed April 9, 1993, and PCT/US91/07542 filed October 15, 1991 mentioned above. It is anticipated that co-administration of at least two autoantigens (and/or fragments of autoantigens) or more broadly at least two other bystander antigens (and/or bystander fragments) will also result in effective suppression of the autoimmune diseases.

In addition, other cytokine and non-cytokine synergists can be conjoined in the treatment to enhance the effectiveness of oral tolerization using bystander antigens alone or bystander antigens plus Th2-enhancing cytokines. Oral use of other cytokine synergists (Type I interferons) has been described in co-pending U.S. Patent Application Serial No. 08/225,372. Non-limiting examples of non-cytokine synergists for use in the present invention include bacterial lipopolysaccharides from a wide variety of gram negative bacteria such as various subtypes of E. coli and Salmonella (LPS, Sigma Chemical Co., St. Louis, MO; Difco, Detroit, MI; BIOMOL Res. Labs., Plymouth, PA), Lipid A (Sigma Chemical Co., St. Louis, MO; ICN Biochemicals, Cleveland, OH; Polysciences, Inc., Warrington, PA); immunoregulatory lipoproteins, such as peptides covalently linked to tripalmitoyl-S-glycarylcysteinyl-seryl-serine (P_3 C55) which can be obtained as disclosed in Deres, K. et al. (Nature, 342:561-564, 1989) or "Braun's" lipoprotein from E. coli which can be obtained as disclosed in Braun, V., Biochim. Biophys. Acta 435:335-337, 1976; and cholera toxin β-chain (CTB) the synergist ability of which have been described (though not in connection with abatement of autoimmune reaction) by Sun, J-B et al., 1994 PNAS (USA) 91, November 1994. LPS is preferred and Lipid A particularly preferred. Lipid A is particularly preferred for use in the present invention because it is less toxic than the entire LPS molecule. LPS for use in the present invention can be extracted from gram-negative bacteria and purified using the method of Galanes et al. (Eur. J. Biochem. 9:245, 1969) and Skelly, R.R., et al. (Infect. Immun. 23:287, 1979). The effective dosage range for noncytokine synergists for mammals is from about 15 μg to about 15 mg per kg weight and preferably 300 μg - 12 mg per kg weight. The effective dosage range for oral Type I interferon for mammals is from 1,000 - 150,000 units with no maximum effective dosage having been discerned.

Oral Use of Th2-Enhancing Cytokines Alone in the Present Invention

According to the present invention, oral, enteral, or by-inhalation administration of Th2-enhancing cytokines is used to suppress autoimmune disease. An example of a Th2 enhancers cytokine is IL-4.

IL-4 can be purified from natural sources (T-cells that normally produce it) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect and mammalian cells, using techniques well-known to those of ordinary skill in the art. In addition, IL-4 is commercially available. The DNA sequence encoding human IL-4 is disclosed in Yokota et al., Proc.Natl.Acad.Sci.USA 83:5894, 1986.

According to the present invention, the route of administration of IL-4 is preferably oral or enteral. The preferred oral or enteral pharmaceutical formulations may comprise, for example, a pill, a liquid or a capsule containing an effective amount of IL-4.

Each oral (or enteral) formulation according to the present invention may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents, and salts, as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures employing solid carriers well-known in the art. Capsules employed in the present invention may be made from any pharmaceutically acceptable material, such as gelatin, or cellulose derivatives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated, such as those described in U.S. Patent No. 4,704,295, issued November 3, 1987; U.S. Patent No. 4,556,552, issued December 3, 1985; U.S. Patent No. 4,309,404, issued January 5, 1982; and U.S. Patent No. 4,309,406, issued January 5, 1982.

Examples of solid carriers include starch, sugar, bentonite, silica, and other commonly used carriers. Further non-limiting examples of carriers and diluents which may be used in the formulations of the present invention include saline, syrup, dextrose, and water.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount, since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof).

In general, when administered orally or enterally, IL-4 may be administered in single dosage form or multiple dosage forms.

Suppression of the clinical and histological symptoms of an autoimmune disease occurs after a specific minimum dosage, which, however, varies according to disease, species of mammal, and cytokine. For oral IL-4, the effective dose range for humans is between about 2,000 and 50,000 international units per day, and preferably about 5,000 and about 20,000 international units per day. The maximum dosage is best ascertained by experimentation. It is anticipated that larger doses are permitted but unnecessary. It is not necessary that a dose of IL-4 be effective by itself if IL-4 (or another Th2-enhancing cytokine) is used in combination with an autoantigen or bystander. Suboptimal doses of Th-2 enhancing cytokines that would potentiate the effect of the bystander or autoantigen can thus be used.

Ascertaining the effective dosage range as well as the optimum amount is well within the skill in the art in light of the information given in this section. For example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose of cytokine (e.g. 500 units of IL-4, progressively) increasing it (e.g. logarithmically) and measuring a biological reaction to the treatment, for example induction of regulatory cells ($CD4^+$ and/or $CD8^+$) as described in Chen, Y. et al., Science, 1994, supra, reduction in class II surface markers on circulating T-cells, and/or by scoring the disease severity, according to well-known scoring methods (e.g., on a scale of 1 to 5, or by measuring the number of attacks, or by measuring joint swelling, grip strength, stiffness, visual acuity, ability to reduce or discontinue medication, etc. depending on the type of disease). The

optimum dosage will be the one having the greatest influence on the biological phenomenon being measured, such as that which causes the greatest induction of regulatory T-cells or the greatest decrease in immune attack cells and/or that which causes the greatest decrease in disease symptoms. An effective dosage range will be one that causes at least a statistically or clinically significant attenuation of at least one symptom characteristic of the disease being treated, or a significant change of a marker (such as the frequency of regulatory or activated T-cells).

Administration of IL-4 may be once daily for a period of time ranging from 30 days to several months (e.g. 3-6) or even years (e.g. 2-6). In fact, therapy may continue indefinitely (unless the obtained benefit does not persist) given the low risk of side effects afforded by the oral route of administration.

Protease inhibitors (such as soybean trypsin inhibitor, aprotinin, antipain) may be added to oral dosage forms containing IL-4 to increase the absorbed amount. In that case, the dosage of IL-4 may be decreased.

Parenteral administration of IL-4 may also be used alone or as an adjunct to oral tolerization therapy but oral IL-4 is preferred because of the systemic effect of parenteral IL-4. Parenteral IL-4 however, is quite effective in suppressing autoimmune disease, as illustrated below. Parenteral dosage for mammals generally can range from about 500 international units of IL-4 to about 1,000,000 international units although the upper limit of this range can best be established by experimentation. It is anticipated that the upper limit will be an amount at which the maximum suppressive effect of parenteral IL-4 is observed (i.e. efficacy will not be lost by using higher amounts but they may be unnecessary). Parenteral administration may take place subcutaneously typically once every other day (without limitation) in a single or in divided doses.

Combination Therapy

It has been surprisingly discovered that the oral (or by inhalation) administration of a bystander antigen in

conjunction with oral or parenteral or by-inhalation administration of IL-4, results in a treatment which suppresses autoimmune reaction, the effect of conjoint therapy being substantially augmented when compared to the effect of each treatment separately.

This combination treatment has been studied in rodents, using the animal model for MS, EAE. The experimental protocol for these studies is disclosed in the Examples below.

Treatment of mice with 1000 international units of IL-4 alone administered orally 5 times before induction of EAE has resulted in reduction in disease incidence and some reduction in maximum clinical score.

Treatment with a combination of oral rat IL-4 (1000 units) and oral tolerization using MBP reduces both disease onset and clinical score, and delays disease onset. In fact, the delay in disease onset was substantially greater (30 days) with the combination treatment than with either IL-4 or MBP alone (21 or 22 days respectively).

It is anticipated that the combination treatment of intraperitoneal IL-4 and oral tolerization with a bystander antigen can show a synergistic suppressive effect on the clinical score of the EAE seen if the doses of each of bystander and Th2 enhancer are reduced to reveal this effect. These treatments clearly have a substantially more pronounced suppressive effect when used in conjunction compared to the effect achieved with either treatment alone.

In practicing the present invention, the bystander antigen is administered essentially as described above. IL-4 is administered in conjunction with the bystander antigen. Oral or enteral administration of IL-4 may be achieved as described above.

Parenteral administration may be via subcutaneous, intramuscular, or intraperitoneal, routes, with subcutaneous being preferred for treatment purposes (although intraperitoneal route was used in the examples below). In the case of parenteral administration, IL-4 may be formulated in sterile saline or other carriers well known in the art, and may

include excipients and stabilizers that are standard in the art.

Ascertaining the optimum regimen for administering both bystander antigens and Th2-enhancing cytokines is well within the skill in the art in light of the information disclosed herein. As is the case with individually administering bystander antigens or Th2-enhancing cytokines routine variation of dosages, combinations, and duration of treatment is performed under circumstances wherein the severity of autoimmune reaction can be measured. Useful dosage and administration parameters are those that result in reduction in autoimmune reaction, including a decrease in number of autoreactive T-cells, or in the occurrence or severity of at least one clinical or histological symptom of the disease.

It is preferred to utilize Th2-enhancing cytokines derived from the same species as the species being treated.

The following examples are illustrative of the present invention and do not limit the scope of the invention.

MATERIALS AND METHODS

In the experiments described below the following materials and methods are used.

Animals. Female Lewis rats 6-8 weeks of age are obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). SJL/J mice, 8 weeks of age are obtained from Jackson Laboratories, Bar Harbor, ME. Animals are maintained on standard laboratory chow and water ad libitum. Animals are maintained in accordance with the guidelines for the Committee on Care of Laboratory Animals of the Laboratory Research Council (Pub. #DHEW:NIH, 85-23, revised 1985).

Antigens and Reagents. Guinea pig MBP and mouse MBP is purified from brain tissue by the modified method of Deibler et al. (Prep. Biochem. 2:139, 1972). Protein content and purity are monitored by gel electrophoresis and amino acid analysis. Histone, hen egg lysozyme and ovalbumin are obtained from Sigma (St. Louis, MO). Peptides are synthesized in the peptide facility of the Center for Neurologic Disease, Brigham and Women's Hospital, and purified on HPLC. The amino acid sequences of the MBP peptides synthesized are: 71-90,

SLPQKSQRSQDENPVVHF (immunodominant encephalitogenic region in rats); the mouse PLP peptides 140-160 (disease-inducing epitope in rats) and 139-153 (disease inducing epitope in mice) were also used.

Mouse IL-4 was obtained from Collaborative Biomedical Products, Bedford, MA.

Induction of Tolerance. For oral tolerance or active suppression, rats are fed 1 mg of MBP dissolved in 1 ml PBS, or PBS alone, by gastric intubation with a 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times at intervals of 2-3 days with the last feeding two days before immunization. When mice are used for experiments, the tolerization regime is substantially the same except that 0.5 mg of MBP and/or 1000 or 5000 units of IL-4 are used for tolerization. Further details for mouse experiments are provided below.

Induction of EAE. For actively induced disease, Lewis rats are immunized in the left foot pad with 25 μg of guinea pig MBP in 50 μl of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 4 mg/ml of Mycobacterium tuberculosis (Difco). When mice are used for experiments, 400 μg of MBP in 0.1 ml of PBS/CFA containing 4 mg/ml of *Mycobacterium tuberculosis*.

Clinical evaluation. Animals are evaluated in a blind fashion every day for evidence of EAE. Clinical severity of EAE is scored as follows: 0, no disease; 1 limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; and 5 death. Duration of disease is measured by counting the total number of days from disease onset (for control rats usually days 10 or 11 after active immunization; for control mice 9 days after immunization) until complete recovery (or death) for each animal.

Histology. Histologic analysis of pathological changes can be performed in animals with induced EAE. Spinal cords are removed on day 15 after adoptive transfer (or disease induction) and fixed with 10% neutral buffered formalin. Paraffin sections are prepared and stained with Luxol fast blue-hematoxylin and eosin, by standard procedures (Sobel et

al., J. Immunol. 132:2393, 1984). Spinal cord tissue is sampled in an identical manner for each animal and numbers of inflammatory foci per section (clusters of >20 or more aggregated inflammatory cells), in parenchyma and meninges are scored in a blinded fashion (Sobel et al., supra).

Statistical analysis. Clinical scales are analyzed with a two-tailed Wilcoxon rank sum test for score samples, chi square analysis is used in comparing the incidence of disease between groups, and comparison of means is performed by using the Student's t-test. For individual experiments, 5 animals are generally used per group.

EXAMPLE 1: Assay for TGF-β Induction

Measurement Of TGF-β Activity In Serum-Free Culture Supernatants. Serum free culture supernatants were collected from antigen-tolerized rats as previously described (Kehri, et al. J. Exp.Med.163: 1037-1050, 1986; Wahl, et al. J.Immunol.145: 2514-2419,1990). Briefly, modulator cells were first cultured for 8 hours with the antigen (50 μl/ml) in proliferation medium. Thereafter cells were washed three times and resuspended in serum-free medium for the remainder of the 72 hour culture, collected, then frozen until assayed. Determination of TGF-β content and isoform type in supernatants was performed using a mink lung epithelial cell line (American Type Culture Collection, Bethesda, MD #CCL-64) according to Danielpour et al. (Danielpour, D., et al. J. Cell. Physiol. 138: 79-86,1989).), and confirmed by a Sandwich Enzyme Linked Immunosorbent Assay (SELISA) assay as previously described (Danielpour et al. Growth Factors 2: 61-71,1989). The percent active TGF-β was determined by assay without prior acid activation of the samples.

This assay can be adapted to test any antigen which is a candidate for use as a bystander. Those antigens, antigen fragments and/or amounts of antigen which produce the highest concentration of TGF-β as measured by this assay can be considered those antigens and/or amounts most suitable for use in the treatment method of the present invention.

Alternatively, a transwell culture system, described below, can be used to indicate the level of TGF-β which is being produced. This culture system measures the production of TGF-β as a function of suppression of cell proliferation.

The appearance of IL-4 and/or IL-10 in culture supernatants of antigen-stimulated cells may also serve as an indicator that the antigen is suitable for use as a bystander. IL-4, IL-10 (and TGF-β) can be assayed by ELIZA using commercially available antibodies to each polypeptide as described in Chen, Y. et al., Science, 1994, supra.

Transwell Cultures. A dual chamber transwell culture system (Costar, Cambridge, MA), which is 24.5 mm in diameter and consists of two compartments separated by a semi-permeable polycarbonate membrane, with a pore size of 0.4 μm, was used. The two chambers are 1 mm apart, allowing cells to be coincubated in close proximity without direct cell-to-cell contact. To measure in vitro suppression of proliferative responses in transwell cultures, 5×10^4 antigen line cells, raised and maintained for example, as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195, 1981), were cultured with 10^6 irradiated (2,500 rad) thymocytes, in 600 μl of proliferation media in the lower well. Spleen cells from orally tolerized rats or controls (fed BSA) were added to the upper well (5×10^5 cells in 200 μl). Spleen cells were removed 7-14 days after the last feeding, and a single cell suspension was prepared by pressing the spleens through a stainless steel mesh. The antigen (50 μg/ml) is added in a volume of 20 μl. Because modulator cells are separated from responder cells by a semi-permeable membrane, they do not require irradiation. In some experiments, modulator cells were added in the lower well together with responder cells, and in these instances modulator cells were irradiated (1,250 rad) immediately before being placed in culture. Proliferation media consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2×10^5 M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1 % HEPES buffer, 1% nonessential amino acids, and 1% autologous serum. Each transwell was performed in quadruplicate. The transwells were

incubated at 37°C in a humidified 6% CO_2 and 94% air atmosphere for 72 hours. After 54 hours of culture, each lower well was pulsed with 4 μCi of [^{3}H]thymidine and at 72 hours split and reseeded to three wells in a round-bottomed 96-well plate (Costar) for harvesting onto fiberglass filters and counting using standard liquid scintillation techniques. Percent suppression = 100 x (1 - Δ cpm responders cultured with modulators/Δ cpm of responders).

EXAMPLE 2: Oral Tolerance Using Bovine-PLP or Mouse MBP

In order to demonstrate bystander suppression, groups of 5-6 female, 7 week old, SJL/J mice (Jackson Labs, Bar Harbor, ME) were immunized with mouse PLP peptide 140-160 on days 0 and 7 and received the following treatments:

GROUPS

1. Fed Histone (0.25 mg/mouse)
2. Fed Mouse MBP (0.25 mg/mouse)
3. Fed Bovine PLP (0.25 mg/mouse) (autoantigen suppression)

The PLP peptide used was the disease inducing fragment 140-160 of bovine PLP. This peptide has the amino acid sequence COOH-PLAYTIGVFKDPHGLWKGLCNH$_2$, representing the foregoing amino acid residues.

Both mouse MBP and bovine PLP were equally effective in down-regulating PLP-peptide-induced EAE when orally administered. A non-specific protein, histone, was ineffective in suppressing EAE when administered orally. Thus, a bystander antigen, in this case mouse MBP, effectively suppressed EAE when orally administered to animals induced for EAE with bovine PLP.

The effects of feeding various peptides to Lewis rats induced for EAE by guinea pig MBP residue nos. 71-90 (the major immunodominant epitope of guinea pig MBP in rats as shown in Example 1 above) were also studied.

EAE was induced by immunizing with 0.25 mg of guinea pig MBP amino acid residue nos 71-90 in Complete Freund's

Adjuvant and the effect of feeding various guinea pig MBP peptides on EAE was examined.

Orally administered whole guinea pig MBP and a 21-40 guinea pig peptide were equally effective in downregulating EAE induced by guinea pig MBP 71-90 as was orally administered 71-90 itself. Guinea pig MBP peptide 131-150 was ineffective in conferring tolerance in rats. Peptides were also fed with STI which prevents their breakdown by gastric juices and enhances their biological effect. DTH responses (to injection of 25 μg of MBP in PBS) to whole MBP were suppressed by feeding MBP or any one of the MBP-peptides 21-40, or 71-90. However, DTH responses to guinea pig MBP peptide 71-90 were only suppressed by feeding either whole MBP or guinea pig peptide 71-90 and were not affected by guinea pig MBP peptide 21-40. This is consistent with the observation that MBP fragment 71-90 does not participate in bystander suppression when fed to mice in which disease had been induced with peptide 71-90. See, Chen, Y. et al., Science, 1994, supra.

The foregoing experiment illustrates an assay system for determining whether an orally administered antigen acts as a bystander suppressor. Whole antigens could have been used instead of fragments.

EXAMPLE 3: **Suppression of EAE in Mice with a Combination of Oral Tolerization using MBP and Oral IL-4**

EAE was induced in SJL/J, 8 week old, female mice by immunizing on day 0 with 400 μg mouse MBP in 0.1 ml of a suspension containing 4 mg/ml *Mycobacterium tuberculosis* (MT), followed by pertussis toxin injection (100 ng/mouse) on days 0 and 2. On days -10, -8, -6, -4, and -2, different groups of mice were treated as follows:

Groups
1. Fed hen egg lysozyme (HEL) (0.25 mg/mouse)
2. Fed mouse IL-4 (1000 international units/mouse)
3. Fed mouse MBP (0.5 mg/mouse)
4. Fed mouse MBP (as in group 3) plus IL-4 (as in group 2).

Animals were monitored for disease onset for 35 days. Animals were scored for signs of disease every day beginning on day 9 on a scale of 0 to 5.

The results were as follows: Substantially simultaneous administration of oral IL-4 with MBP clearly augmented the suppressive effect of MBP in terms of disease incidence, and delay of onset. At 1000 international units/mouse/feeding, IL-4 alone had a suppressive effect on disease incidence similar to that of MBP when fed alone.

This type of experiment can be used to assess efficacy of treatment with Th2-enhancing cytokine alone or combination treatment with Th2 enhancer and bystander. Dosages can be adjusted to observe the effect(s) of dosage variation.

EXAMPLE 4: Suppression of EAE in Mice with a Combination of Oral Tolerization using MBP and Oral IL-4

EAE is induced in SJL/J, 8 week old, female mice by immunizing on day 0 with 400 μg mouse MBP in 0.1 ml of a suspension containing 4 mg/ml *Mycobacterium tuberculosis* (MT), followed by pertussis toxin injection (100 ng/mouse) on days 0 and 2. On days -10, -8, -6, -4, and -2, different groups of mice are treated as follows:

Groups

1. Feed mouse IL-4 (5000 international units/mouse)
2. Feed mouse MBP (0.5 mg/mouse)
3. Feed mouse MBP (as in group 2) plus IL-4 (as in group 1).

Animals are monitored for disease onset for 35 days. Animals are scored for signs of disease every day beginning on day 9 on a scale of 0 to 5.

This experiment will demonstrate that at the foregoing dose, feeding IL-4 alone significantly delays the onset of disease, decreases fatality, and/or reduces the mean and maximum clinical scores. Furthermore, feeding IL-4 at the foregoing dose, in combination with MBP, significantly augments the suppressive effect observed when MBP alone is fed.

EXAMPLE 5: Suppression of PLP-Induced EAE in Mice with a Combination of Oral Tolerization using MBP and Oral IL-4

EAE is induced in SJL/J, 8 week old, female mice by immunizing on day 0 with 100 μg mouse PLP 139-151 peptide in 0.1 ml of a suspension containing 4 mg/ml *Mycobacterium tuberculosis* (MT), followed by pertussis toxin injection (100

ng/mouse) on days 0 and 2. On days -10, -8, -6, -4, and -2, different groups of mice are treated as follows:

Groups
1. Feed mouse IL-4 (5000 international units/mouse)
2. Feed mouse MBP (0.5 mg/mouse)
3. Feed mouse MBP (as in group 2) plus IL-4 (as in group 1).

Animals are monitored for disease onset for 35 days. Animals are scored for signs of disease every day beginning on day 9 on a scale of 0 to 5.

This experiment will demonstrate that at the foregoing dose, feeding IL-4 alone significantly delays the onset of PLP-induced disease, decreases fatality, and/or reduces the mean and maximum clinical scores. Furthermore, feeding IL-4 at the foregoing dose, in combination with the pure bystander antigen MBP, significantly augments the suppressive effect observed when MBP alone is fed.

Example 6: **Suppression of Adjuvant Arthritis in Mice with a Combination of Oral Tolerization using Type II Collagen and IL-4**

Arthritis is induced in male DBA/1 Lac J mice by subdermal immunization with 300 μg bovine Type II Collagen in a suspension containing 4 mg/ml MT. On days -14, -12, -10, -8, -6, -4, and -2, different groups of mice are treated as follows:

Groups
1. Feed mouse IL-4 (5000 international units/mouse)
2. Feed mouse collagen type II (0.5 mg/mouse)
3. Feed mouse collagen type II (as in group 2) plus IL-4 (as in group 1).

Animals are monitored for onset of arthritis for 60 days. Beginning on day +10, animals are scored for signs of arthritis on a scale of 0 to 4. The arthritis score for each animal is the sum of the score for each of the four paws.

This experiment will show that at the foregoing dose, feeding IL-4 alone significantly delays the onset of disease symptoms and/or reduces the mean and maximum clinical scores. Furthermore, feeding IL-4 at the foregoing dose, in combination with type II collagen, significantly augments the suppressive effect observed when collagen alone is fed.

Example 7: Oral IL-4 Alone and in Combination with Insulin in Suppression of NOD Diabetes

Groups of 4-week-old non-obese diabetic (NOD) mice (3 animals per group) are treated as follows:

1. Fed ovalbumin (1mg/mouse)
2. Fed equine insulin (1mg/mouse)
3. Fed mouse IL-4 (5000 units/mouse)
4. Fed insulin (as in group 2) and IL-4 (as in group 3)

Mice are fed 10 times on alternate days. About 3 weeks after the experiment the following parameters are assessed: insulitis and/or time of diabetes onset.

This experiment will show that at the foregoing dose, feeding IL-4 alone significantly reduces the above clinical indicators. Furthermore, feeding IL-4 at the foregoing dose, in combination with insulin, significantly augments the suppressive effect observed when insulin alone is fed.

EXAMPLE 8: Suppression of EAE in Mice with a Combination of Oral Tolerization using MBP and Intraperitoneal Administration of IL-4

EAE was induced in SJL/J, 8 week old, female mice by immunizing on day 0 with 400 μg mouse MBP in 0.1 ml of a suspension containing 4 mg/ml *Mycobacterium tuberculosis* (MT), followed by pertussis toxin injection (100 ng/mouse) on days 0 and 2. Different groups of mice are treated as follows:

Groups
1. Injected mouse IL-4 intraperitoneally (5000 international units/mouse/injection) on day 0 and day 3.
2. Fed mouse MBP (0.5 mg/mouse) on days -10, -8, -6, -4, and -2.
3. Fed mouse MBP (as in group 2) plus injected IL-4 (as in group 1).

Animals were monitored for disease onset for 35 days. Animals were scored for signs of disease every day beginning on day 9 on a scale of 0 to 5.

This experiment demonstrated that at the foregoing dose, injecting IL-4 alone significantly reduced the incidence, delayed the onset of disease and reduced the clinical scores. The magnitude of this effect was such that it was impossible

to assess whether parenteral administration of IL-4 could augment the protection afforded by oral MBP alone, which also showed comparable suppression of autoimmune reaction (as measured by the foregoing indicators).

EXAMPLE 9: **Suppression of EAE in Mice with a Combination of Oral Tolerization using MBP and Intraperitoneal Administration of IL-4**

EAE is induced in SJL/J, 8 week old, female mice by immunizing on day 0 with 400 μg mouse MBP in 0.1 ml of a suspension containing 4 mg/ml *Mycobacterium tuberculosis* (MT), followed by pertussis toxin injection (100 ng/mouse) on days 0 and 2. Different groups of mice are treated as follows:

Groups
1. Feed mouse MBP (0.3 mg/mouse) on days -10, -8, -6, -4, and -2.
2. Inject mouse IL-4 intraperitoneally (500 international units/mouse)
3. Inject mouse IL-4 intraperitoneally (1000 international units/mouse)
4. Inject mouse IL-4 intraperitoneally (2000 international units/mouse)
5. Feed mouse MBP (as in group 1) plus IL-4 (as in group 2).
6. Feed mouse MBP (as in group 1) plus IL-4 (as in group 3).
7. Feed mouse MBP (as in group 1) plus IL-4 (as in group 4).

IL-4 injections will be administered as in Example 8.

Animals are monitored for disease onset for 35 days. Animals are scored for signs of disease every day beginning on day 9 on a scale of 0 to 5.

This experiment will demonstrate that at the indicated reduced doses, parenteral administration of IL-4, in combination with MBP, significantly augments the suppressive effect observed with oral administration of MBP alone.

WHAT IS CLAIMED:

1. A method for suppressing autoimmune reaction in a mammal diagnosed with a T-cell mediated or T-cell dependent autoimmune disease, the method comprising administering to said mammal: (i) via the oral or enteral route, an amount of a bystander antigen and (ii) an amount of a noninterferon polypeptide having Th2-enhancing cytokine activity, the amounts of said antigen and said polypeptide being effective in combination in reducing said autoimmune reaction substantially more than the amount of either said antigen or said cytokine administered alone.

2. The method of claim 1 wherein the amounts of said antigen and said polypeptide are substantially more effective in suppressing said reaction in combination compared to the suppressive effects achieved by administering each of said antigen and said polypeptide alone.

3. The method of claim 1 wherein said polypeptide is selected from the group consisting of IL-4 and fragments thereof having Th2-enhancing cytokine activity.

4. The method of claim 1, wherein said polypeptide is IL-4.

5. The method of claim 4, wherein said IL-4 is derived from the same species as said mammal.

6. The method of claim 1, wherein said polypeptide is administered orally.

7. The method of claim 1 wherein said mammal is a rodent and said disease is a rodent model for multiple sclerosis.

8. The method of claim 1 wherein said mammal is a human and said disease is multiple sclerosis.

9. The method of claim 7 wherein said bystander antigen is selected from the group consisting of myelin, myelin basic protein (MBP), proteolipid protein (PLP), tolerogenic fragments thereof and combinations of at least two of the foregoing.

10. The method of claim 8 wherein said bystander antigen is selected from the group consisting of myelin, myelin basic protein (MBP), proteolipid protein (PLP), tolerogenic fragments thereof and combinations of at least two of the foregoing.

11. The method of claim 1 wherein said disease is selected from the group consisting of rheumatoid arthritis, collagen-induced arthritis, and adjuvant-induced arthritis, and said bystander antigen is selected from the group consisting of Type I collagen, Type II collagen, Type III collagen, tolerogenic fragments thereof and combinations of two or more of the foregoing.

12. The method of claim 1 wherein said disease is selected from the group consisting of Type I diabetes and said bystander antigen is selected from the group consisting of GAD, glucagon, insulin, tolerogenic fragments thereof, and combinations of two or more of the foregoing.

13. The method of claim 1 wherein said disease is selected from the group consisting of uveoretinitis and animal models therefor and said bystander antigen is selected from the group consisting of S-antigen, interphotoreceptor retinoid binding protein (IRBP), fragments thereof, and combinations of two or more of the foregoing.

14. A method for reducing an autoimmune reaction in a mammal diagnosed with a T-cell mediated or T-cell dependent autoimmune disease, the method comprising:

administering to said mammal a noninterferon polypeptide having Th2-enhancing cytokine activity in an amount

effective to reduce at least one clinical or histological symptom of said disease.

15. The method of claim 14 comprising administering a polypeptide selected from the group consisting of IL-4 and fragments thereof having Th2-enhancing cytokine activity.

16. The method of claim 14 wherein said mammal is a human.

17. The method of claim 14 wherein said polypeptide is IL-4 administered via the oral or enteral route.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04512

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/17, 38/19, 38/20, 38/26, 38/28, 38/39.
US CL :424/85.1, 85.2, 184.1; 514/2, 3, 8,; 530/303, 308, 350, 351, 356.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 85.2, 184.1; 514/2, 3, 8,; 530/303, 308, 350, 351, 356.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 5,190,750 (OTSUKA ET AL.) 02 March 1993, see entire document.	14 ---- 1-13, 15-17
X -- Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 178, issued July 1993, Rapoport et al., "Interleukin-4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice," pages 87-99, see entire document.	14-15, 17 ---- 1-13, 16
X,P --- Y,P	THE JOURNAL OF CLINICAL INVESTIGATION, Vol. 94, issued October 1994, Rizzo et al. "Interleukin-2 treatment potentiates induction of oral tolerance in a murine model of autoimmunity," pages 1668-1672, see entire document.	1-2, 6, 13-14 ---- 3-5, 7-12, 15-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 JULY 1995

Date of mailing of the international search report

18 JUL 1995

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 18D, issued 07 April 1994, Nelson et al., "ß-interferon enhances oral tolerance to myelin proteins in experimental autoimmune encephalomyelitis," page 447, abstract V785, see Abstract.	1-17
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, Vol. 89, issued January 1992, Miller et al., "Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor ß after antigen-specific triggering," pages 421-425, see entire document.	1-17
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 176, issued November 1992, Khoury et al., "Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor ß, Interleukin 4, and prostaglandin E expression in the brain," pages 1355-1364, see entire document.	1-17
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 151, No. 12, issued 15 December 1993, Miller et al., "Epitopes of myelin basic protein that trigger TGF-ß release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression," pages 7307-7315, see entire document.	1-17
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 142, No. 3, issued 01 February 1989, Lider et al., "Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein," pages 748-752, see entire document.	1-17
Y	AUTOIMMUNITY, Vol. 15, Supplement, issued 1993, H. L. Weiner, "Treatment of autoimmune diseases by oral tolerance to autoantigens," pages 6-7, see entire document.	1-17
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 174, issued October 1991, Miller et al., "Antigen-driven bystander suppression after oral administration of antigens," pages 791-798, see entire document.	1-17
Y	SCIENCE, Vol. 259, issued 26 February 1993, Weiner et al., "Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis," pages 1321-1324, see entire document.	1-17

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRITISH JOURNAL OF RHEUMATOLOGY, Vol. 30, Supplement 2, issued 1991, N. A. Staines, "Oral tolerance and collagen arthritis," pages 40-43, see entire document.	1-17
Y	AUTOIMMUNITY, Vol. 16, issued 1993, Thompson et al., "Suppression of collagen induced arthritis by oral administration of type II collagen: Changes in immune and arthritic responses mediated by active peripheral suppression," pages 189-199, see entire document.	1-17
Y	SCIENCE, Vol. 261, issued 24 September 1993, Trentham et al., "Effects of oral administration of type II collagen on rheumatoid arthritis," pages 1727-1730, see entire document.	1-17
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Vol. 88, issued November 1991, Zhang et al., "Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin," pages 10252-10256, see entire document.	1-17
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 144, No. 5, issued 01 March 1990, Nussenblatt et al., "Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen," pages 1689-1695, see entire document.	1-17
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 151, No. 10, issued 15 November 1993, Gregerson et al., "Oral tolerance in experimental autoimmune uveoretinitis," pages 5751-5761, see entire document.	1-17
Y	NEUROLOGY, Vol. 44, No. 4, issued April 1994, Al-Sabbagh et al., "Beta interferon enhances oral tolerance to MBP and PLP in experimental autoimmune encephalomyelitis," page A242, abstract 465P, see Abstract.	1-17
Y	ANNUAL REVIEWS OF IMMUNOLOGY, Vol. 12, issued April 1994, Weiner et al., "Oral Tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens," pages 809-837, see entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04512

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Vol. 91, No. 23, issued 08 November 1994, H. L. Weiner, "Oral Tolerance," pages 10762-10765, see entire document.	1-17
Y,P	SCIENCE, Vol. 265, issued 26 August 1994, Chen et al., "Regulatory T Cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis," pages 1237-1240, see entire document.	1-17
Y,P	EUROPEAN JOURNAL OF IMMUNOLOGY, Vol. 24, issued November 1994, Fishman-Lobell et al., "Different kinetic patterns of cytokine gene expression *in vivo* in orally tolerant mice," pages 2720-2724, see entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04512

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04512

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, drawn to methods for suppressing an autoimmune response using bystander antigen and non-interferon polypeptides (IL-4).

Group II, claim 11, drawn to a second method, a method for treating rheumatoid arthritis.

Group III, claim 12, drawn to a third method, a method for treating Type I diabetes.

Group IV, claim 13, drawn to a fourth method, a method for treating uveoretinitis.

Group V, claims 14-17, drawn to a fifth method, a method for reducing autoimmune responses using Th2 enhancing peptides.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods of Groups I-V are directed to methods utilizing different reagents and treating autoimmune diseases which differ in their pathology and etiology and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.